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Survival motor neuron – motor neuron insurance for a whole lifespan?

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Abstract

The SMN (survival motor neuron) gene plays an important role in ontogenesis and its dysfunction leads to immaturity of skeletal muscles and motor neurons in the spinal cord. As a result of SMN mutations the affected cells die and clinical symptoms of spinal muscular atrophy (SMA) develop. Physiologically, SMN together with gemins is part of a multiprotein complex of particular importance to motor neuron development. Since the SMN gene is necessary for normal motor neuron maturity, a question arises whether its expression is preserved in postnatal life or finishes with the end of ontogenesis.

To answer this question we examined expression of SMN and gemins 2, 3 and 4 in spinal cords of Wistar rats at age 1-350 days using immunofluorescence and immunohistochemical methods.

In the examined animals expression of SMN appeared in neurons in 20-day old rats and increased with animal age. In rats aged 30-350 days SMN immunoreactivity was similar in all the examined animals. The same phenomenon was observed in assessment of gemin expression. Our study revealed that in rat spinal cord expression of SMN and gemins 2, 3 and 4 is present through a whole animal lifespan and not only in motor but also in sensory and autonomic neurons.

Key words: *SMN, gemins, protein expression, spinal cord, rat.*

Introduction

Among many neurological diseases spinal muscular atrophy (SMA) is one of the most dramatic illnesses. It is characterized by immaturity of spinal motor neurons and muscle cells. The immature cells do not attain maturity and die, which is manifested clinically as weakness and atrophy of skeletal muscles. There are several forms of SMA. The most severe form is called Werdnig-Hoffman disease (group 1) and its symptoms are observed directly after delivery (floppy children). It is characterized by disturbances in milk sucking and weak motor activity of newborns. In group 2, the course of the disease is more benign. In group 3 with clinical onset in young adults, SMA course is the least devastating.

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At the end of the twentieth century it was discovered that forms 1, 2, and 3 of SMA are caused by mutations in exon 7 of the Survival Motor Neuron (*SMN*) gene mapped on chromosome 5q11.2-q13.3 [9]. In humans, the *SMN* gene possesses two copies – telomeric (*SMN1*) and centromeric (*SMN2*). SMA develops as a result of mutations only in the SMN1 gene. Lack of SMN-delta7 mRNA in SMN2 causes that mutations in exon 2 are not pathogenic. In other mammals only one copy of the *SMN* gene exists. An elegant study performed by Rochette *et al*. [13] did not reveal presence of *SMN2* on sub-human monkeys. The authors cited above suggested that *SMN* duplication appeared over 3 million years ago, before division into anthropoids and *Homo sapiens*. The SMN-gemin complex is present not only in mammals [3]. Presence of *SMN* protein and gemins 2, 3 and 5 has been found in *Drosophila* [4].

Physiologically, SMN binds to 7 multifunctional proteins called gemins (gemins 2-8), forming an oligomeric complex [12,15]. Functions of gemins are different. Gemin 2 stabilizes the SMN-gemin complex [12] while gemins 4 and 5 transmit the oligomeric complex from and to the cell nucleus [1,6]. Gemins 2 and 3 are colocalized with SMN protein and are present in all cell compartments (cell body, dendrites, axons and growth cones [18]), while gemins 6 and 7 are localized only in the cytoplasm.

The SMN-gemin complex together with small nuclear ribonucleoproteins takes part in RNA splicing. It is known that mutations in the *SMN* gene lead to alterations in SMN oligomerization and formation of SMN-gemin complex [7], resulting in disturbances in proliferation, migration and development of nerve cells [17]. In SMN-depleted mice, increased proliferation and morphological changes in nerve cells and developmental disturbances in stem cells were observed [14]. The transgenic animals also revealed regionally selected abnormalities in CNS morphology manifested as decreased proliferation and cell density in the hippocampus [17]. But the *SMN* gene acts not only in early ontogenesis. In experimental SMA it was shown that postnatal activity of SMN protein was neuroprotective [8,11,18,20]. These data raise some new questions:

1. How long during a lifespan is the *SMN* gene active?
2. Are only anterior horn motor neurons protected by SMN?
3. Does the *SMN* gene protect motor neurons only from spinal muscular atrophy or also from other disorders proceeding with degeneration of spinal motor neurons such as ALS?

Material and methods

The material consisted of spinal cords from 27 Wistar rats at the age of 1-350 days (9 groups composed of 3 rats at the age of 1, 10, 20, 30, 60, 150, 200, 250 and 350 days).

In rat spinal cords fixed in formalin and embedded in paraffin, expression of SMN and gemin 2, 3 and 4 was assessed in light and immunofluorescence (only SMN and gemin 3) microscopy. Immunohistochemistry for light microscopy was performed according to the avidin-biotin-peroxidase method. Tissue slides were dehydrated in alcohol, pre-treated with heat retrieval using a microwave for 3 × 10 min in 10 mM citrate buffer (pH 6.0), and immunostained with primary antibodies against SMN (Santa Cruz Biotechnology, 1 : 250), gemin 2 (Santa Cruz Biotechnology, 1 : 500), gemin 3 (Santa Cruz Biotechnology, 1 : 1000), and gemin 4 (Santa Cruz Biotechnology, 1 : 1000) using Goat F(ab)2 Fragment anti-mouse IgG-biotin (Beckman Counter, 1 : 1500) and Goat F(ab)2 Fragment anti-rabbit IgG-biotin (Beckman Counter, 1 : 1500) respectively and diaminobenzidine as the chromogen.

For fluorescent microscopy double immunolabelling of SMN and gemin 3 was performed. The first part of the procedure was the same as the procedure of single immunohistochemistry but then simultaneous incubation with two primary antibodies (Abs), monoclonal mouse anti-gemin 3 Ab and polyclonal rabbit anti-SMN Ab, was performed. Next, the sections were washed and incubated for 1 h at 37°C with secondary antibodies: goat anti-mouse Alexa Fluor 594 (Invitrogen – Molecular Probes, 1 : 100) and goat anti-rabbit Alexa Fluor 488 (Invitrogen – Molecular Probes, 1 : 100). Then, they were washed in PBS, dried and mounted with Vectashield Mounting Medium (Vector Laboratories Inc) for fluorescence microscopy. Sections were analysed and captured with an Optiphot-2 Nikon microscope (Japan) equipped with the appropriate filters and a DS-L1 Nikon camera (Japan).

Specificity of the immune reactions was verified by performing a "negative control" staining procedure with primary antibodies omitted in the incubation mixture.

Apart from immunohistochemistry, computer-based image processing methods were applied for

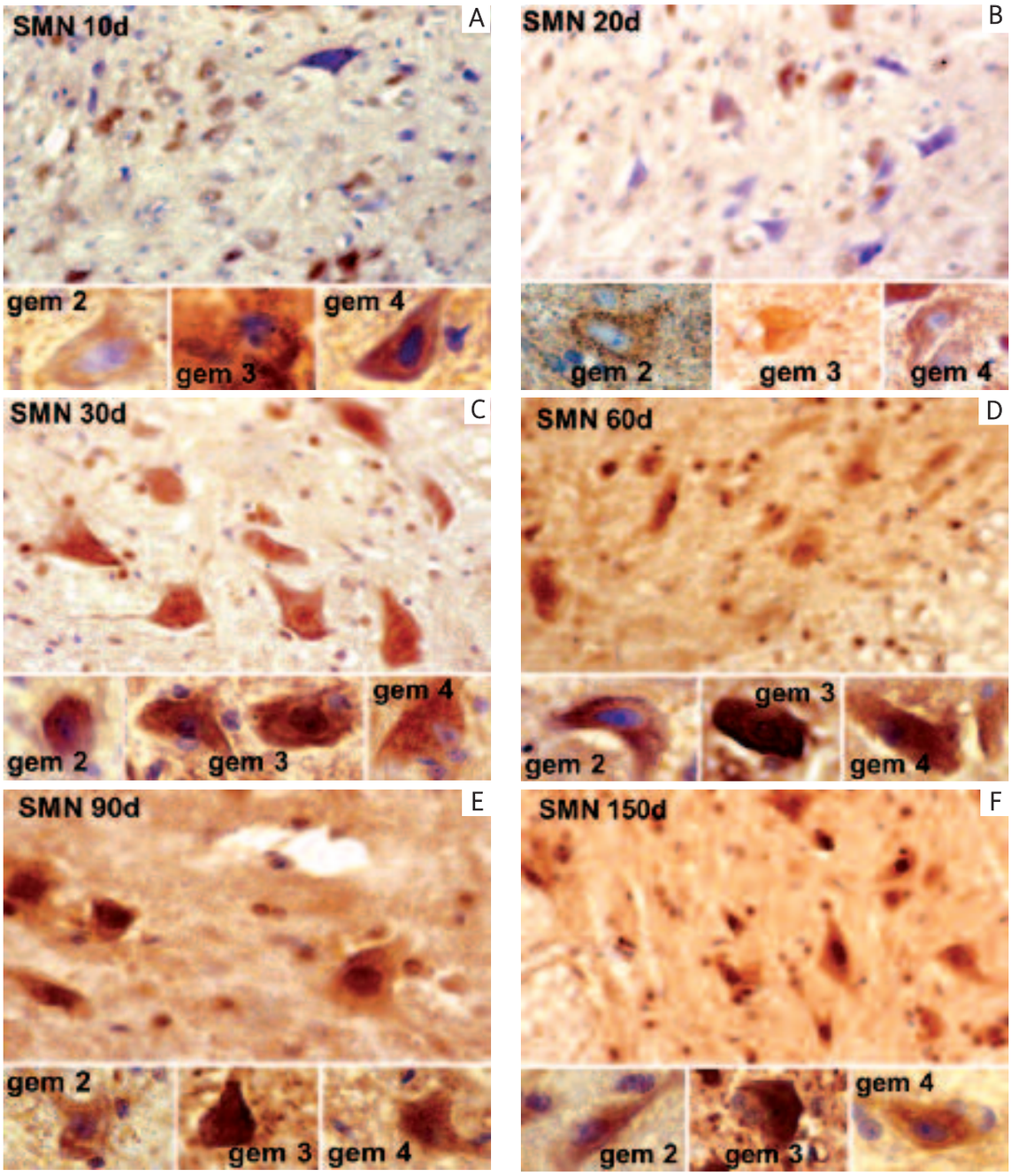

Fig. 1. Expression of SMN and gemins 2, 3 and 4 in rat anterior horn cells. SMN – expression of SMN protein, gem – expression of gemin, d – days old, org. magn. – original magnification. **A, B)** Lack or very weak SMN expression in the anterior horn neurons. Visible weak expression of gemin 2 and more intense immunoreactivity of gemin 3 and 4; SMN – org. magn. 100×; gem 2, 3, 4 – org. magn. 200×. **C, D, E, F)** In all figures visible evident cytoplasmic and nuclear expression of SMN in neurons. A part of glial cells also reveals SMN immunoreactivity. Immune reactions to gemin 2, 3 and 4 show their pronounced cytoplasmic and nuclear expression. SMN – org. magn. 100×; gem 2, 3, 4 – org. magn. 200×.

graphical visualization of the SMN staining intensity within ventral horn areas of the investigated sections. The image processing software Scion Image (Scion Image for Windows Scion Corporation) was used for the analysis. Sections were converted to greyscale, calibrated on an 8-bit scale and surface plot profiles were performed. The height of plots pointed to the level of intensity of the immune reaction.

Results

Expression of SMN in rats at the age of 10 days was absent or very weak and only a part of cells revealed the positive immune reaction within neuronal cytoplasm (Fig. 1A). In 20-day old rats SMN-immunopositive neurons were more numerous and the immune reaction was more pronounced than in the younger animals (Fig. 1B). Nuclear localization of SMN was observed in rats starting from the 30th day of life (Fig. 1C). In rats at the age of 30-350 days pronounced SMN immunoreactivity was present in all the examined animals (Figs. 1D-F, Figs. 2A-C).

Graphic visualization of the SMN immunolabel within rat ventral horns (shown in Figs. 1D-F and Figs. 2A-C) is demonstrated in Figs. 5A-C, in which the height of plots indicates the level of intensity of the immune reaction. Data obtained in the graphic method confirmed our results from the immunohistochemical studies and showed increase of intensity of the SMN-immunoreactive signal in sections derived from older rats at the age of 30-350 days in comparison to younger rats at the age of 1-20 days.

Assessment of gemins 2, 3 and 4 revealed immunoreactivity pattern of their expression similar to SMN. Analogous to SMN immune reaction, nuclear localization of gemin 2 was also observed in rats starting from the 30th day of life (Fig. 1C).

In rats at the age of 10 days we noted a very interesting phenomenon. Contrary to very weak immune reactions to SMN and gemin 2 in that period, expression of gemins 3 and 4 was very prominent and found both in neuron cytoplasm and nucleus (Fig. 1A).

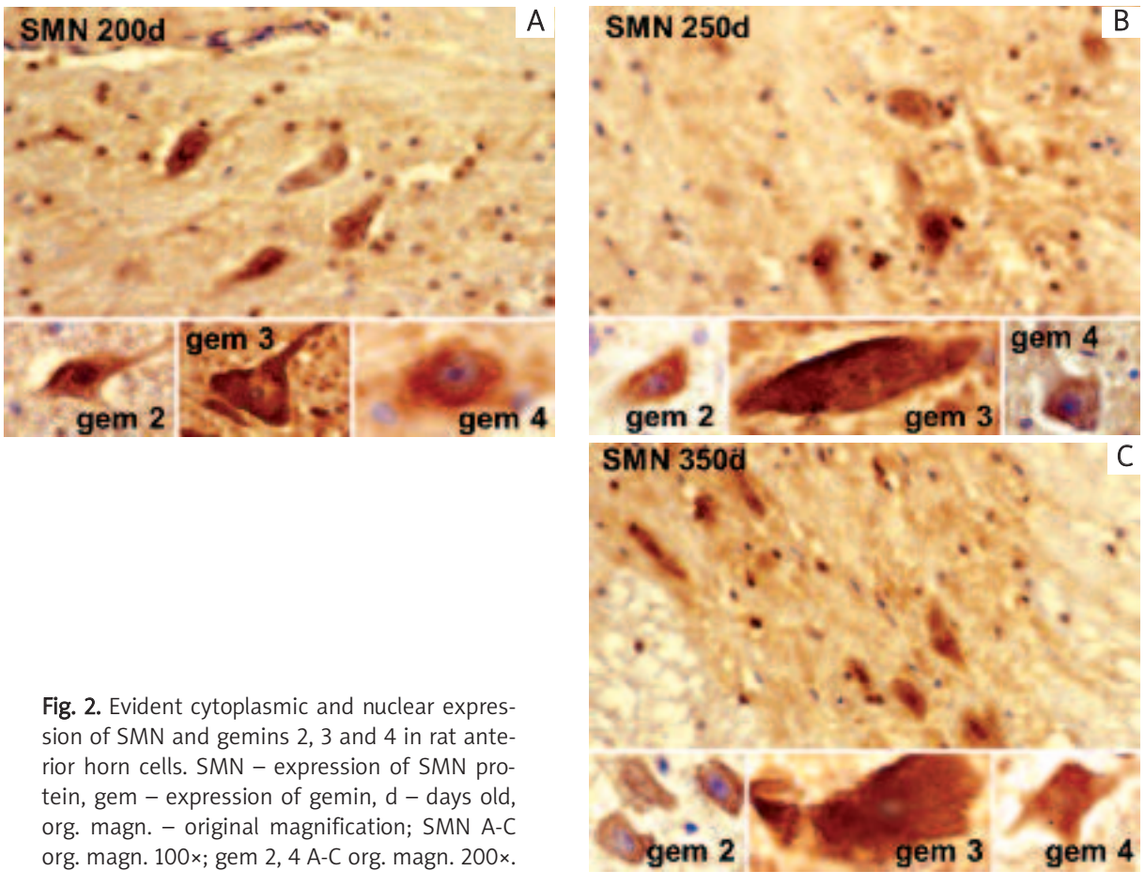

Fig. 2. Evident cytoplasmic and nuclear expression of SMN and gemins 2, 3 and 4 in rat anterior horn cells. SMN – expression of SMN protein, gem – expression of gemin, d – days old, org. magn. – original magnification; SMN A-C org. magn. 100×; gem 2, 4 A-C org. magn. 200×.

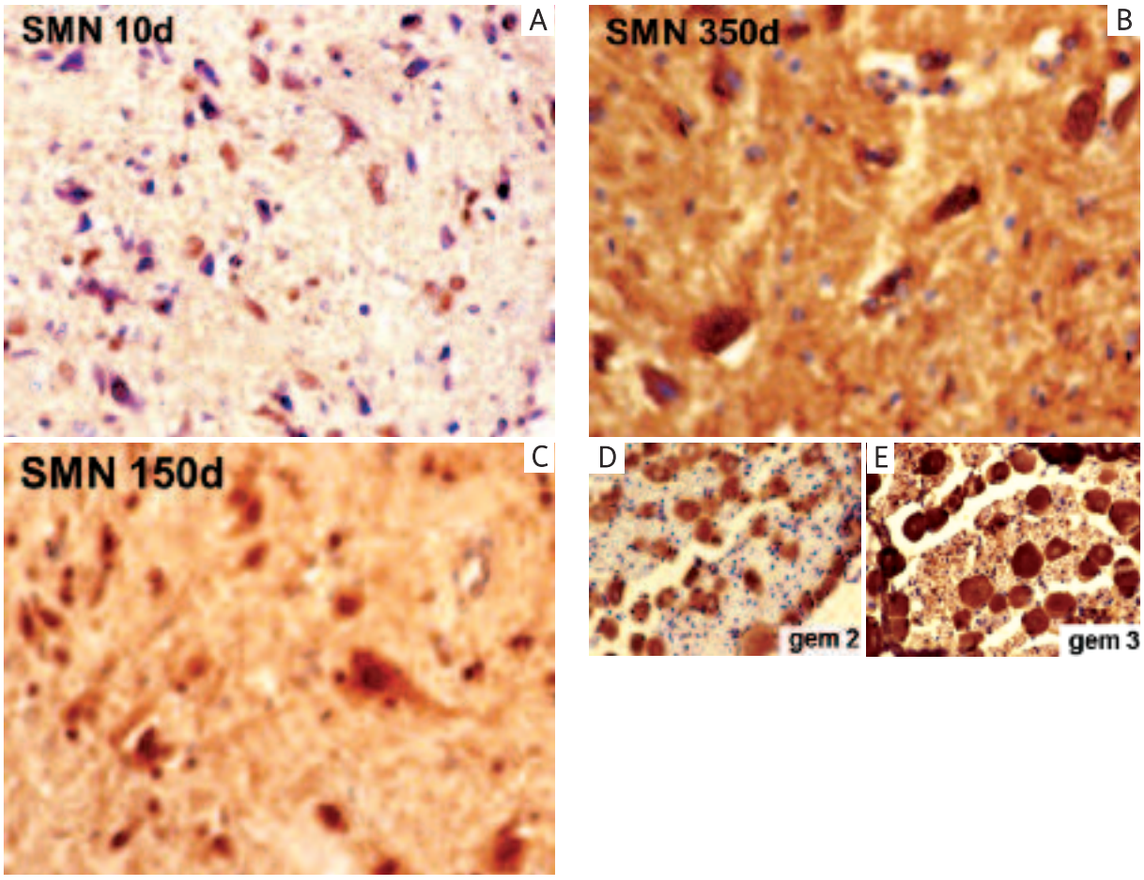

Fig. 3. Expression of SMN and gemins 2, 3 and 4 in rat neurons. SMN – expression of SMN protein, gem – expression of gemin, d – days old, org. magn. – original magnification. **A)** Lack or weak SMN expression in posterior horn neurons in 10-day old rat; org. magn. ×100. **B)** Pronounced expression of SMN in posterior horn neurons in 350-day old rat; org. magn. ×100. **C)** Evident expression of SMN in neurons of posterior horn in 150-day old rat; org. magn. ×100. **D, E)** 150-day old rats; strong cytoplasmic immunoreactivity of sensory ganglion neurons to gemin 3 (D) and gemin 4 (E), org. magn ×200.

The immune label for SMN and gemins was observed in neurons both in anterior and posterior spinal cord horns (Figs. 3A-C) as well as in vegetative neurons within lateral horns in the thoracic spinal cord (Fig. 3D) and in neurons in sensory spinal ganglia (Fig. 3E).

In the immunofluorescent method, colocalization of gemin 3 with SMN protein in the same neurons was demonstrated (Figs. 4A-B). The cell membrane was especially very intensively decorated.

Discussion

In the literature there are scarce data concerning activity and expression of the *SMN* gene and they refer mainly to experimental animals. In animals SMN was found in the CNS, liver, kidneys, lungs and muscles [7]. The protein expression was maximal in the embryonic and early postnatal period and decreased after ontogenesis [2,7]. In humans, expression of SMN in fetuses and adults was also investigated [5,16]. In spite of these studies, the dynamics of SMN expression during the lifespan remain unknown.

In our experimental rat material involving a long period of the animal's life (from birth to old age), very weak expression of SMN protein was already visible in a part of neurons in rats at the age of 20 days and the expression increased with the ani-

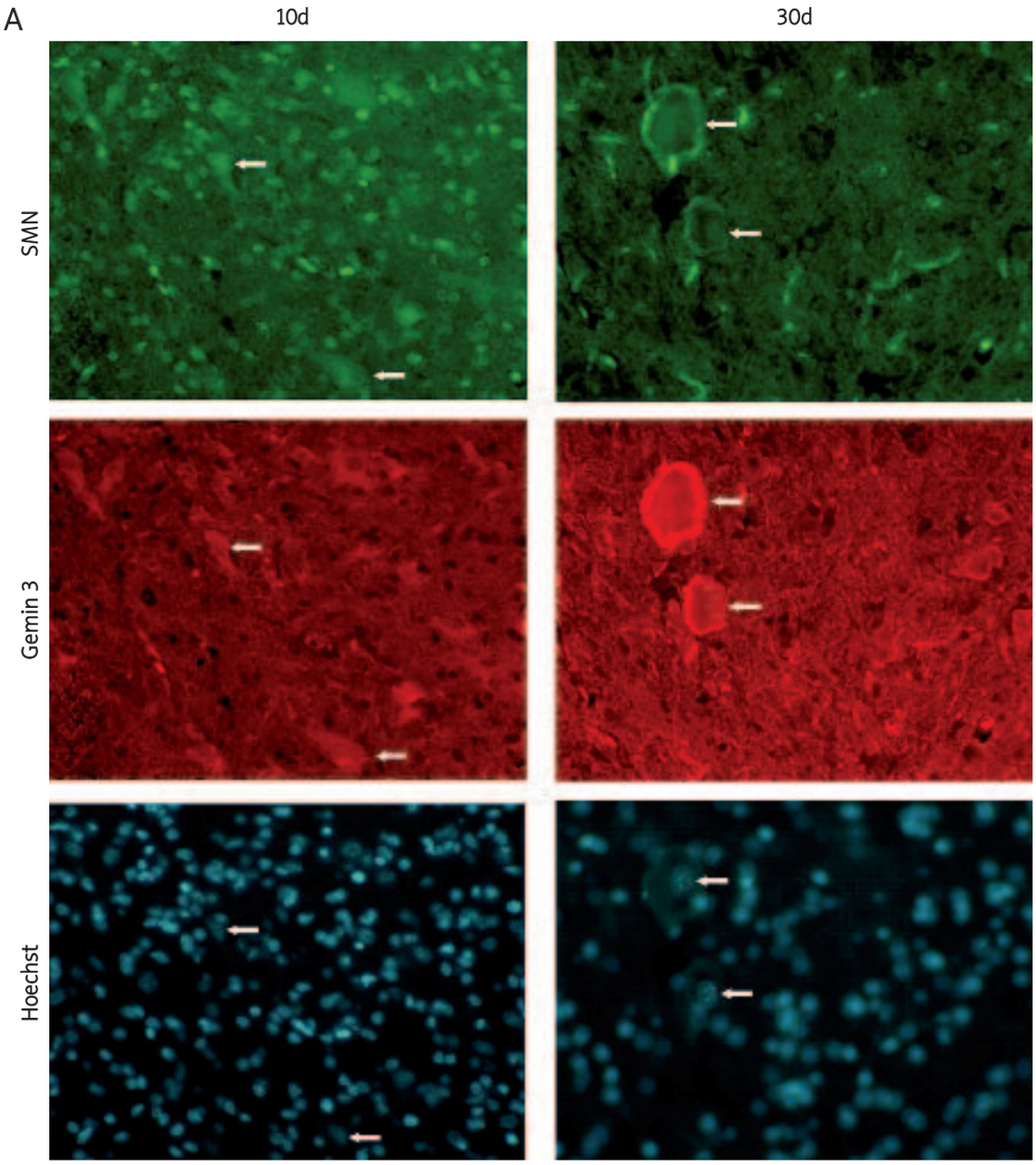

Fig. 4. A, B) Immunofluorescence deposits of SMN (green, upper microphotographs) and gemin 3 (red, middle microphotographs) in the cells within the ventral horn of rat spinal cords. Animals of different age (A – 10, 30 days; B – 150, 250 and 350 days). Morphology of cell nuclei – bisbenzimide (Hoechst, blue, lower microphotographs); org. magn. ×100. The level of immunosignal increased until 30 days of lifetime and then persisted. Please note the colocalization of SMN and gemin 3 in the same spinal cells and the presence of granular condensed puncta of immunomaterial in the cytoplasm close to the cell membrane.

B

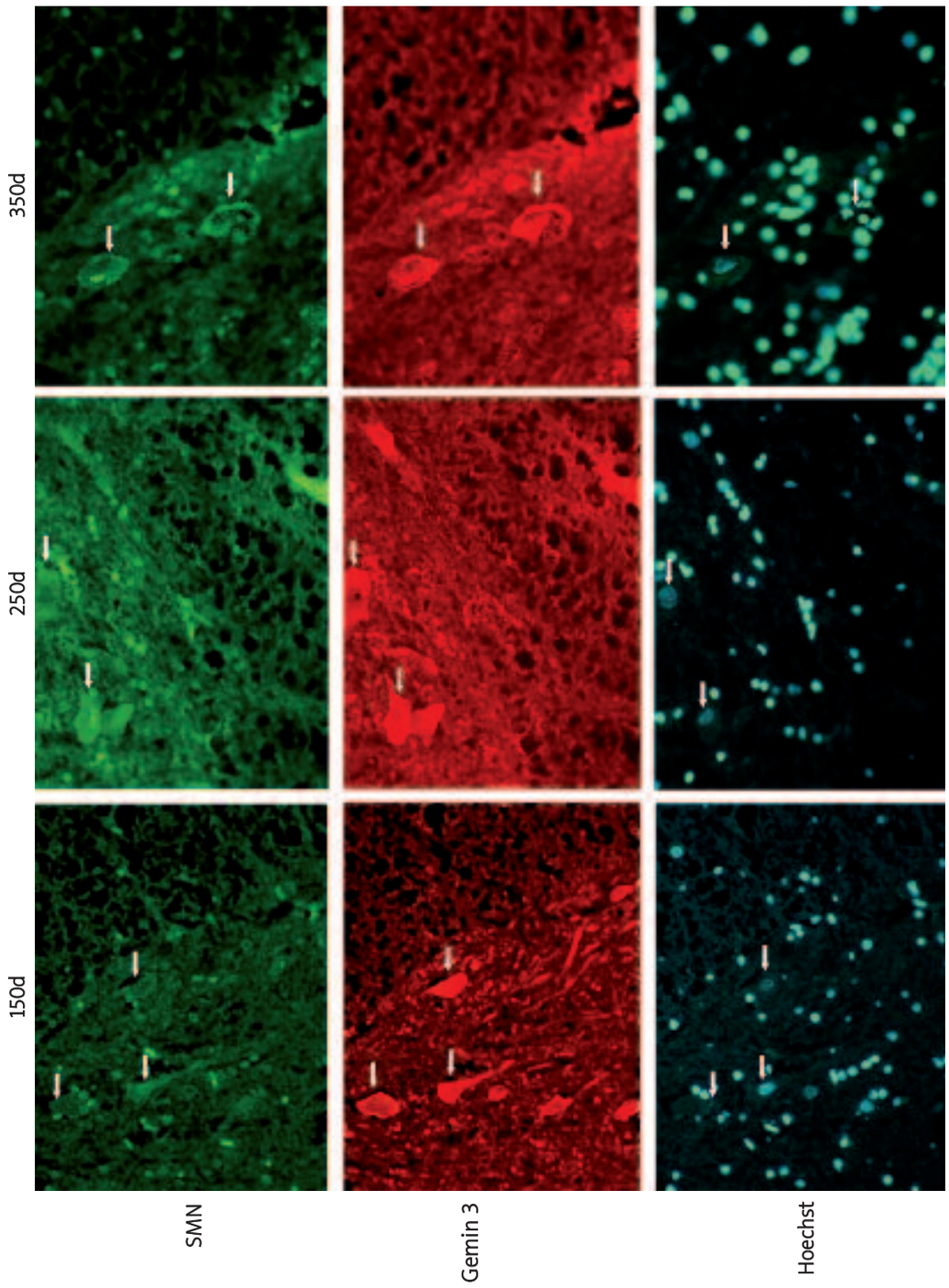

Fig. 4. Cont.

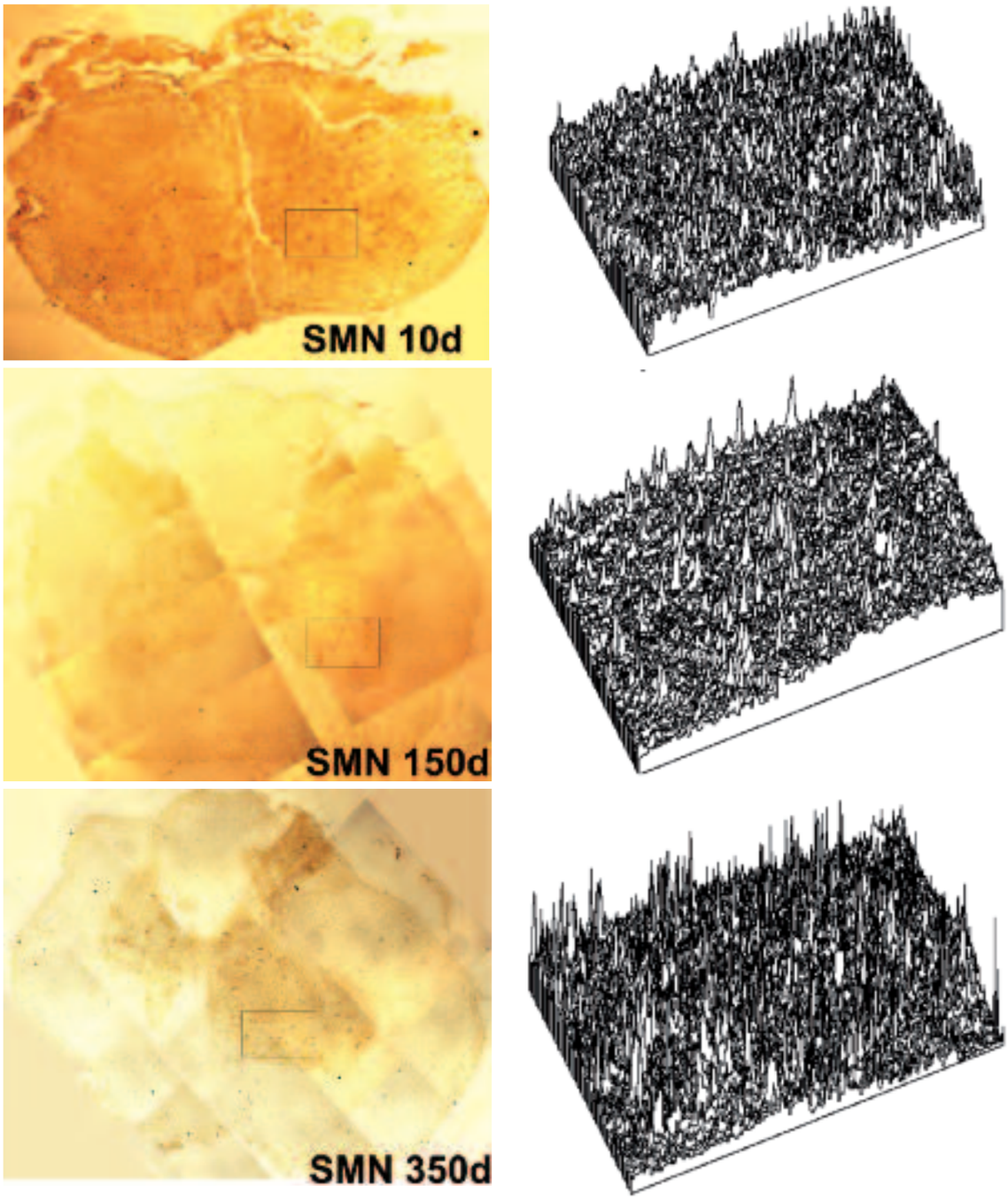

Fig. 5. Photomicrographs of representative sections of rat spinal cords immunostained for SMN (left panel). The intensity of staining of ventral horn regions (frames) is shown as the height of the surface plot profiles (right panel).

mal's age. It was confirmed both in light and immunofluorescent studies. Surface plot profiles of the SMN immunolabel in rat ventral horn areas also showed enhancement of the immunostaining intensity in the postnatal period. Contrary to humans, in neonatal rats and mice both anterior and posterior horn cells are immature. Our results suggest that the immune expression of SMN is parallel with cell maturity. The clinical improvement after postnatal induction of SMN expression observed in the mice model of SMA [8] might be connected with this phenomenon and confirms our supposition. Also nuclear localization of SMN found in our material in rats at the age of 30 days and older may indicate cell "maturity".

Our observations of SMN expression in rats aged 30-350 days revealed its various immunoreactivity in spinal cord neurons and glial cells. This suggests that SMN expression may be dependent on the level of the protein synthesis and associated with the functional stage of cells. Various SMN expression may also be dependent on neuron size and individual differences among the cells, although it is not clear whether such relationships really exist. Graphical visualization of the SMN immunostaining data in cells in rat ventral horns showed the intensification of individual pixel labelling. These results demonstrate that during maturation not only the number of cells immunoreactive to SMN (motoneurons, interneurons) increases but also intensity of the immune signal. The data point to the presence of SMN in many cells during the lifespan and indicate that in rats the *SMN* gene is probably active during the whole lifespan of the animals.

Estimation of gemin expression in rats at the age of 10 and 20 days revealed pronounced immunoreactivity of gemin 3 and 4 while expression of gemin 2 was poor. Since SMN expression at that period was also very weak or absent, it may suggest that in the SMN complex gemins 3 and 4 appear earlier than SMN protein and gemin 2. Moreover, since gemin 2 stabilizes the SMN complex, perhaps full activity of the complex occurs later, after complex stabilization. Poor reactivity to gemin 2 observed in 350-day old rats may also suggest that its expression decreases with aging.

Nuclear localization of SMN and the investigated gemins was observed in rats starting from the 30th day of life. This finding may indicate that transport of the SMN complex to the nucleus by gemin 4 [10] and from the nucleus by gemin 5 [16] (not investigated in our material) probably is already present.

The previously observed colocalization of gemin 3 with SMN protein in neurons [18] was confirmed in our immunofluorescent study. It was interesting that in our investigation the cell membrane was especially very intensively decorated. It is not clear what was responsible for it – maybe numerous immunoreactive receptors?

The name Survival Motor Neuron suggests connection of the gene/protein only with motor neurons. But in our material apart from motoneurons alpha and gamma, also interneurons in spinal posterior horns expressed SMN and gemins. In addition, neurons in Clarke's columns and even sympathetic nerve cells in thoracic lateral horns and intervertebral ganglia were evidently immunopositive to SMN and the investigated gemins. This finding implies that the *SMN* gene may influence not only motoneurons but also sensory and vegetative neurons. In other words, all nerve cells in rat spinal cord may be under the influence of the *SMN* gene.

Since expression of the *SMN* gene essential for neuron survival is preserved after ontogenesis and its mutations result in SMA development, there is a hypothesis that the gene may play a protective role in other neurodegenerative disorders involving spinal cord neurons such as amyotrophic lateral sclerosis (ALS). The first reports which seem to confirm that supposition have been published. It was demonstrated that in a rat ALS model with SOD-1 mutation, SMN protein acts neuroprotectively [19]. Its neuroprotective influence was also observed in vitro in neuroblastoma cells with SOD-1 mutation [20]. But this interesting hypothesis requires further investigations.

Conclusions

1. In rat spinal cord the immune expression of SMN and gemins 2, 3, and 4 was present from the early postnatal period to old age.
2. The expression of the investigated proteins was observed both in motoneurons alpha and gamma, as well as in interneurons and glia.
3. In rat spinal cord the immune expression of SMN and gemins 2, 3, 4 was also present in sensory and autonomic neurons.
4. Since the SMN and gemin expression was found in rat spinal cord neurons it means that not only

mice but also the rat experimental model may be useful in SMA studies.

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